CHEMISTRY OF THE CROSSLINKING OF COLLAGEN DURING TANNING

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ABSTRACT

The materials commonly used for crosslinking collagen as part of the process of converting animal hides into leather fall into three main groups: mineral tannages, aldehyde tannages, and "vegetable" tannages. The most important mineral crosslinking agents are hydrated basic chromium III sulfate complexes. These compounds form extended polynuclear coordination complexes containing hydroxol, oxo, and sulfato bridges into which ionized carboxyl groups on collagen enter readily as coordinating ligands accomplishing crosslinking. On pH adjustment and partial drying, highly stable complexes are formed with oxo bridges predominating and protein amide groups entering the coordination complex. The aldehyde tannages proceed through aldehyde condensation reactions with collagen amino groups to give alpha-hydroxyamines which can condense with other collagen amine groups to effect crosslinking. The vegetable type tanning agents, whether natural plant extracts or synthesized, are complex, high molecular weight polyhydroxy compounds that do not rely on crosslinking as such to be effective. Their effectiveness appears to depend on other properties. This and additional information concerning these commercial tannages are reviewed.

INTRODUCTION

The hide matrix from which leather is made is composed primarily of the protein collagen ordered in microcrystalline

helical units. The crystalline structure is stabilized by a large number of hydrogen bonds between peptide nitrogens and opposite carbonyl groups in the helix. These microcrystallites are then connected by a secondary amorphous structure in which polar and nonpolar side chains participate and covalent crosslinking is established. This has been discussed thoroughly in earlier papers in this symposium.

Native hide collagen is chemically and biologically inert when dry but is stiff, brittle, and relatively useless. However, when wet, it is subject to biological attack and is hydrothermally unstable. The crystallites melt at 58°C in pure water and as low as 37°C in salt solutions.

The purpose of tannage is primarily to increase hydrothermal stability of the crystallites, secondarily to increase biological inertness, and finally, to improve the utility of the hide's physical properties.

A simple empirical shrink temperature test has been used in most tanning research as the criterion of hydrothermal stability. To interpret the literature one must understand precisely what this test measures. When native or tanned hide collagen absorbs water, it swells and extends in length. As the swollen collagen is heated in water it retains its dimensions until a critical temperature is recorded as the shrink temperature. It had been suggested (Garrett and Flory, 1956) that the shrink temperature corresponded to the melting point (Tm) of the water solvated collagen crystallites. This concept was explored at the Eastern Regional Research Center (ERRC) (Witnauer and Fee, 1957), where the temperatures at which this shrinkage of native and tanned collagen occurred were measured over a range of moisture and other solvent contents. This data (Figure 1) supports four postulates critical to understanding and analyzing the effect of tannages on the hydrothermal stability of collagen and the meaning of shrink temperature.

(1) Native collagen crystallite melting points are depressed by nonreactive solvents as predicted by thermodynamics of a crystalline polymer-solute system.

(2) The melting point Tm decreases with increasing amounts of dissolved solute according to an equation of the form

$$\Delta Tm = Tm^{\circ} - Tm = A\ Tm\ v_s(1 - \chi v_s)$$

where Tm° is the melting point of the polymer at zero solute content, v_s is the volume fraction of the solute, and A and χ are constants for a given polymer-solute pair. The magnitude of A

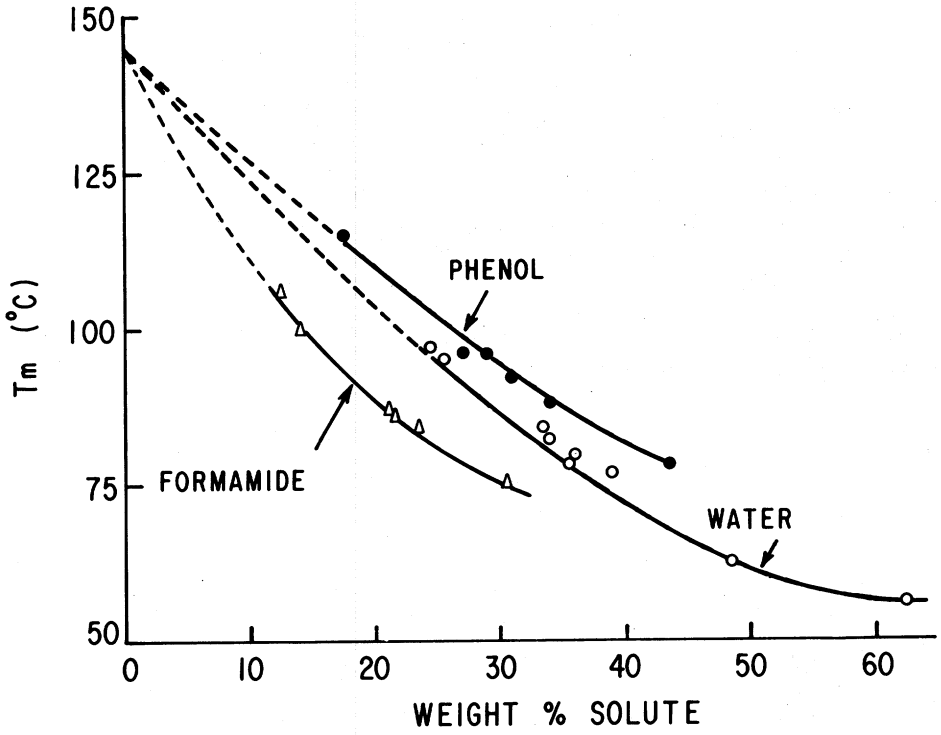


Figure 1. Melting Point (Tm) Depression of Native Collagen.

depends on the energy change in the crystalline polymer transition being observed and the sign and magnitude of χ depend on the energy of interaction between the polymer and solute (Flory, 1953).

(3) The maximum melting point depression with a given solute is limited by the extent of solubility (the last points for each of the three solutes in Figure 1). Further additions of (or immersion in) the solute will not further decrease the melting point.

(4) Native collagen has an intrinsic (extrapolated) melting point (Tm°) of 145°C, when completely "dry" (no solute present). Thus if a treatment or "tannage" of collagen does not alter the native collagen crystallites, the observed Tm° should be 145°C. Conversely, observed values of Tm° other than 145°C indicate that the structure of the collagen crystallites has been changed by the treatment.

Interpretation of observed increases in Tm° as indicating increased crosslinking and decreases in Tm° as indicating a decrease in crosslinking in the collagen crystallites, along with the four postulates discussed above, leads to the following interpretation of the data of Kawamura et al. (1975) in Figure 2. Figure 2 shows the melting point depression curves (with water as the solute) for untanned, chrome-tanned, and vegetable (mimosa)-tanned calfskin. As in the previous data, Tm for untanned hide extrapolates to give a Tm° of 145-150°C at 0% H_2O. However, the Tm° for chrome-tanned skin is distinctly higher than 145°C, indicating cross linking. Tm° for the vegetable-tanned skin is definitely lower than 145°C. This fact, plus the slope of the curve, suggests that the vegetable tannin is actually destabilizing the helix at low moisture levels

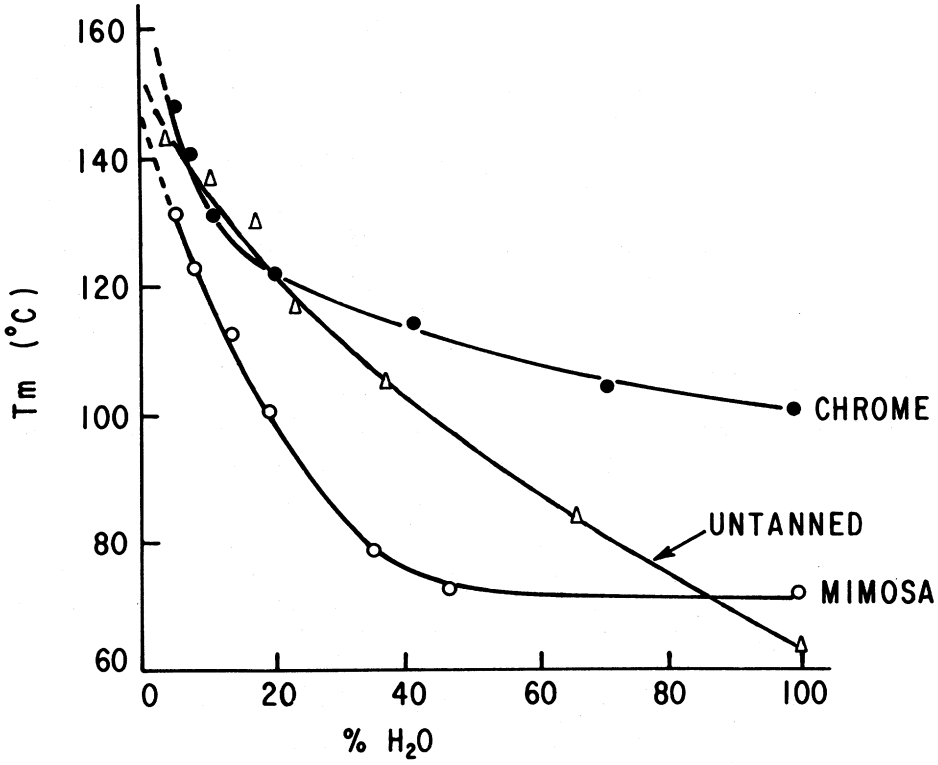


Figure 2. Melting Point (Tm) Depression of Tanned and Untanned Calfskin.

(probably by competing for hydrogen bonding sites normally involved in crosslinking). The fact that vegetable tannins are practical commercial tannages appears due to their ability to limit the effective solubility of water. In this example, the vegetable-tanned hide is hydrothermally stable up to 75°C, even in aqueous solutions, while the shrinkage temperature of the untanned hide falls to 60°C when immersed in water.

The evidence in the literature on the mechanism of hydrothermal stabilization of the major tannages, using this approach, is summarized in (Table I). The changes in the intrinsic crystallite melting points (ΔTm°) are referred to native collagen. The observed polymer-water interaction energy constants χ of equation 1, are expressed here as B values. $B = \chi RT/V_s$, where V_s is the molar volume of water. The B values reflect the degree of curvature in the curves of Figure 1 and 2. Positive values of B indicate that the polymer is a poor solvent for water. Negative values indicate high solvency power. Relative torsional modulus measurements (Witnauer and Fee, 1957) of untanned, vegetable-tanned, and formaldehyde-tanned hides led to the postulation that an increase in modulus indicated increased crosslinking. Estimations of the crosslinks remaining (by tensile modulus measurements after destroying the hydrogen bonding in the crystallites) gave the data in the last column for aldehyde tannages (Cater, 1963). Unfortunately, none of these methods has been applied to compare directly all of these important commercial tannages. However, we can conclude that aldehyde and chrome tannages involve crosslink formation while vegetable tannages do not.

The depression of Tm° and a large positive interaction coefficient for vegetable tannages suggest that they act as solutes, competing with and blocking the effectiveness of water as a solute and thereby providing hydrothermal stability. Formaldehyde, on the other hand, results in an increased Tm° and a negative B, suggesting a crosslinking mechanism. The only relevant study including glutaraldehyde (Cater, 1963) suggests substantial crosslinking.

The surprisingly limited data on chrome tanning shows both a large increase in crystallite melting point, indicating crosslinking and a large positive B suggesting that the high degree of hydrothermal stability imparted by this tannage is due to operation of both stabilizing mechanisms: reducing the solute effect of water and providing additional crosslinking.

CHEMISTRY OF CROSSLINKING REACTIONS

The types of functional groups available for reaction and their relative molar amounts are shown on the top row of Table II. The

TABLE I

Effect of Tannage on Cowhide and Solute Activity of Water

System	$\Delta Tm°$[a] (°C)	B[a] (cal/cc)	Modulus[a] (Relative)	Crosslinks[b] (#/10^5g)
Untanned	0 (ref.)	+2	1.0 (ref.)	0.2
Vegetable Tanned	-25	+35	1.5	
Formaldehyde Tanned	+6	-9	5.0	5
Chrome Tanned	+ (<20)[c]	+(large)[c]	-	-
Glutaraldehyde Tanned	-	-	-	10

[a]Witnauer and Fee, 1957. $\Delta Tm°$ = $Tm°$ observed - $Tm°$ of untanned collagen. B is the energy of interaction of the polymer-water system = $\chi RT/V_s$ (see text).

[b]Cater, 1963.

[c]Kawamura, 1975.

peptide and hydroxyl groups are present in greatest number. They are polar, but not ionized, and form hydrogen bonds with natural vegetable and synthetic (syntan) type tannins.

Mineral tannages crosslink through coordination with the relatively abundant carboxyl ions on collagen and provide the most hydrothermally stable and commercially important tannages. While chrome, aluminum, zirconium and combinations of these are used, chrome tanning is by far the most important.

Aldehyde tannages form stable covalent crosslinked compounds through the relatively limited number of amine groups available.

Chromium Tanning

Chrome tanning is generally carried out by adding the hide collagen to an aqueous solution of 33% basic trivalent chromium

TABLE II

Functional Groups Important In Tanning

	P|-C(=O)-OH	P|-NH_2	P|-C(=O)-NH-R	P|-OH
Relative Number	3	1	19	3
Type of Tannage	"Mineral" Chrome Zirconium Alum	Aldehyde Formaldehyde Glutaraldehyde	Vegetable Syntans	
Type of Bonds Formed	Coordinate	Covalent	Hydrogen	

sulfate. By using a combination of the latest gel permeation, gel electrophoresis, ion exchange chromatography, and spectroscopic techniques, Slabbert (1975) established that there are at least 10 ionic and neutral complexes in a 33% basic chrome sulfate solution. The structures of eight of these were determined along with the relative amounts of each. The six compounds present in highest concentration are shown in Figure 3.

The simplest ion, Cr^{+3}, with six coordinated waters, was present as 9% of the total chrome. The routes of formation of the complexes resulting from replacement of water with sulfate ion (left side of the figure) or by hydroxide ion (right side of the figure) are indicated.

The salient features of the route of formation of the most abundant species, the $^{+2}$ charged binuclear complex with a bidentate sulfate bridge, are the replacement of H_2O by $SO_4^=$ in $Cr(H_2O)_6^{+3}$ to give the +1 monosulfate ion, the formation of the third complex in a series of steps in which OH^- replaces H_2O in a pair of ions, and condensation to form the olate bridged binuclear structure with monodentate sulfate groups. One sulfate group then rotates into the plane of the other, displacing it from the coordination complex, to form the bidentate sulfate bridge.

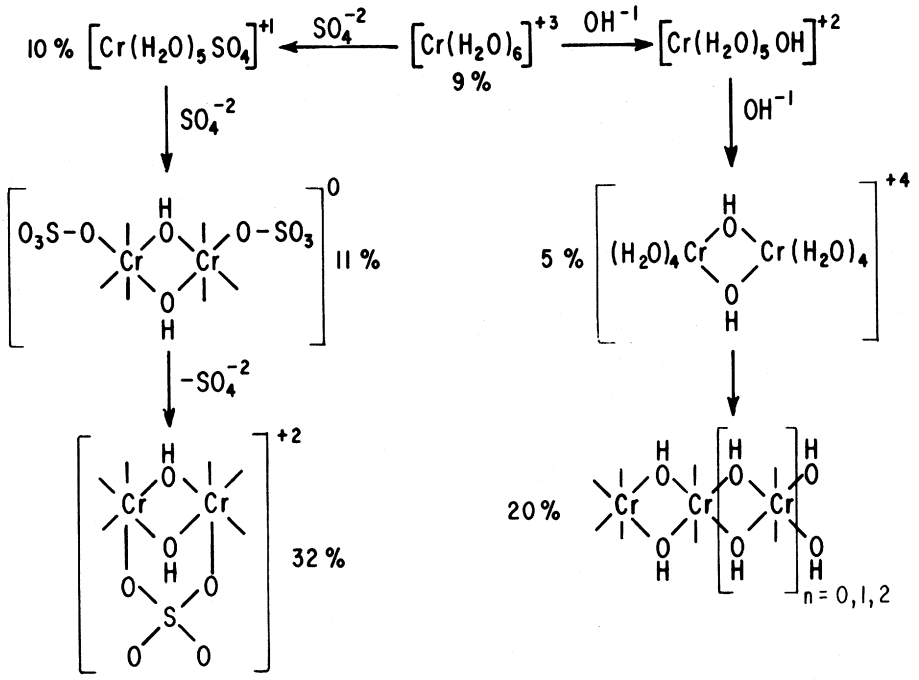


Figure 3. Composition of Typical Chromium Sulfate Tanning Solution (33% basic; 0.4 molar in Cr-III).

This is, of course, a purely descriptive and oversimplified mechanism, but it gives an idea of the type of reactions possible consistent with experimental evidence and the thermodynamics and stereochemistry of chrome complexes as recently reviewed (Irving, 1974).

In Figure 4, we show some of the same types of reactions that can take place as the carboxylate ion attached to the collagen enters these complexes. The carboxylate group can displace water from the $[Cr(H_2O)_6]^{+3}$ ion (9% abundance) to form monodentate bonds, as shown in the upper equation, or bidentate coordinate bonds with binuclear complexes like the one shown in the lower equation. Two mechanisms by which crosslinking can occur (Figure 5) are:

(1) Straightforward entry of two carboxylate ions into the same chrome complex.

(2) Olation involving elimination of water and formation of a linkage between two complexes.

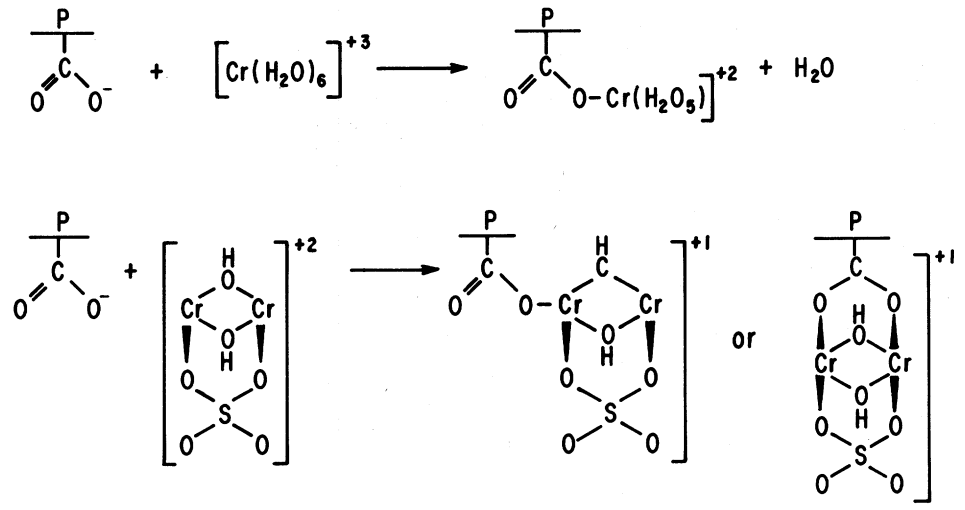


Figure 4. Complex Formation with Protein Carboxyl Groups.

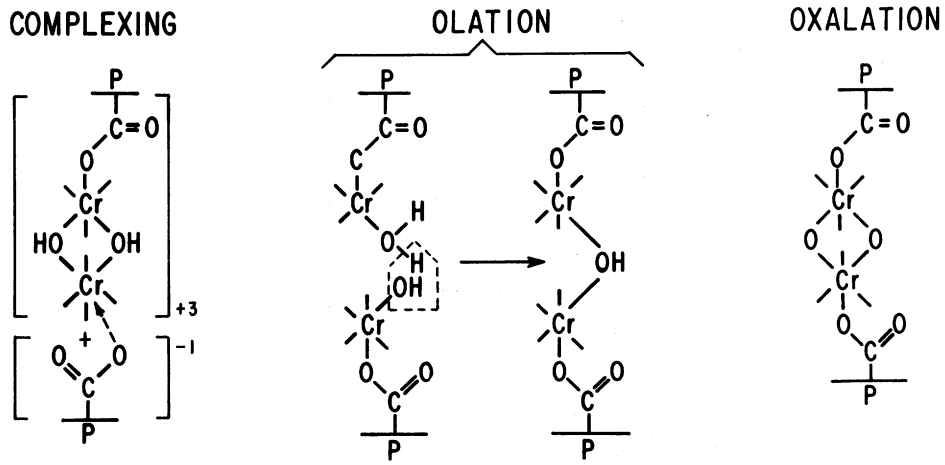


Figure 5. Crosslink Formation from Chromium Complexes.

The olation reaction is favored by increasing the alkalinity of the reaction mixture. As the reactions proceed and multinuclear complexes form with multiple olate bridges, hydronium ions are released and highly stable oxalate bridges are formed, as shown on the right hand side of Figure 5. While not shown here for the sake of simplicity, there is evidence that bidentate sulfate groups remain in the final complex after curing and drying. Apparently they play a role in improving stability of the complexes.

Similar coordination complexes are involved in other mineral tannages. All of these complexes can be reversed or modified by acids, salts, strong bases, and chelating agents. Chromium compllexes, while more difficult to form, have the advantage over other complexing cations of reacting much more slowly in these ligand replacement reactions and therefore producing leather more serviceable in use. Chromium^{+3} is also unique in its resistance to oxidation.

Aldehyde Tanning

The most important collagen functional group involved in aldehyde crosslinking is the amino group. Some potential reactions which need to be considered are shown in Figure 6. Formation of a methylol or substituted methylol derivative is certainly the first

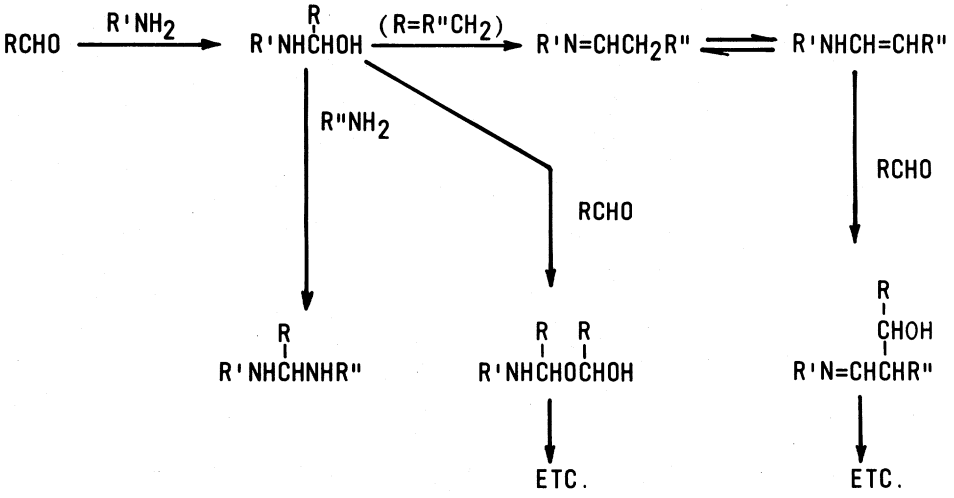
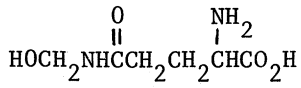


Figure 6. Aldehyde-Amine Reactions.

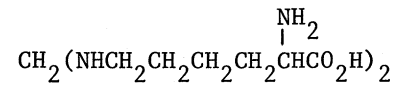
step. Several alternatives are then possible. Apparently direct substitution of an amino group for the hydroxyl is a possibility. This leads directly to a crosslink in a protein, but a hydrolyzable one. Addition of more aldehyde can take place and, except for formaldehyde, Schiff's base formation can take place. In this latter case, further steps lead to condensation reactions of the type shown here, which have recently been proposed for glutaraldehyde crosslinking. While other aldehydes have been investigated for their tanning ability, only formaldehyde and glutaraldehyde are used industrially. Since these two aldehydes crosslink by different mechanisms, they are discussed separately.

The results of early studies on the reactions of protein with formaldehyde have been reviewed (Mellon, 1958; Walker, 1964). Most of these studies were based on the use of model compounds, and none resulted in the isolation of characterized products that were directly related to potential crosslinks in proteins. The most extensive of these studies were those of Fraenkel-Conrat (Fraenkel-Conrat et al., 1945; Fraenkel-Conrat and Olcott, 1948), who demonstrated which functional groups of proteins could react, either alone or in combination, with formaldehyde. These groups included primary amino and amido groups, secondary amide groups of the peptide bond, guanidino groups, imidazoyl groups, indolyl groups, thiol groups, and phenolic groups. Of these, all but the indolyl and thiol groups are found in collagen and are thus potenially capable of taking part in crosslinking. However, since the industrial process is carried out under relatively mild conditions of pH and temperature and in relatively dilute solution, definite limitations are placed on the types of reactions that can be involved. The reactions of interest must also be reversible since simple washing of formaldehyde-tanned collagen with water slowly extracts formaldehyde, while acid hydrolysis accompanied by steam distillation results in a quantitative recovery. This is not true for all protein, but it is true for collagen. Therefore, only those types of products which are formed under mild conditions and which are hydrolyzable should be considered.

A study of the reactions of formaldehyde with wool keratin probably indicates most clearly the types of compounds formed with collagen, even though this protein has a different amino acid composition (Caldwell and Milligan, 1972). The formaldehyde treatment was carried out under extremely mild conditions using ^{14}C-formaldehyde, and the product was enzymatically hydrolyzed. It is interesting that histidine and tyrosine were recovered quantitatively and thus were probably not involved in the reaction. Several ^{14}C-labelled products were detected, but only two were sufficiently separated to permit some judgment to be made concerning their identities. They were reported to be δ N-hydroxymethylglutamine (I) and ε-N,ε-N'-methylenedilysine (II). The latter represents a crosslink.



I



II

The formation of this latter product involves simple addition of a lysinyl residue side chain amino group to formaldehyde, followed by condensation of the resulting N-methylol derivative with another lysinyl residue side chain. Acid hydrolysis of the product leads to recovery of the formaldehyde and lysine.

The crosslinks formed in collagen as a result of formaldehyde tannage are probably of this simple and hydrolytically unstable type, indicated in this study on wool (Caldwell and Milligan, 1972). Perhaps further effort should be given to an extension of this approach to elucidate the structures formed in formaldehyde tannage of collagen.

Glutaraldehyde, unlike formaldehyde, irreversibly crosslinks collagen and other proteins. The crosslinks that are formed have not been fully characterized but progress is being made toward achieving this. Before these crosslinks are discussed, however, some discussion of glutaraldehyde itself is felt to be needed since uncertainty still exists concerning the nature of this crosslinking agent. Proposals have been made in the past (Richards and Knowles, 1968) and continue to be made (Mansan, Puzo, and Mazarquil, 1975) that the reactive species is a condensation polymer of glutaraldehyde. That this is most certainly not the case has been demonstrated by a number of studies (Hardy, Nicholls, and Rydon, 1969; Korn, Feairheller, and Filachione, 1972; Whipple and Ruta, 1974; Blass, Verriest, Lean, and Weiss, 1976). A variety of derivatives of glutaraldehyde, which have been proposed as being present in aqueous solutions of the reagent, are shown in Figure 7, along with glutaraldehyde itself. Perhaps all of these are present, although various studies have proposed specific combinations. The important fact is that all are in equilibrium with glutaraldehyde and glutaraldehyde itself is probably the reactive species.

Of all the crosslink characterization studies made thus far, the most recent (Hardy, Nicholls, and Rydon, 1976) is perhaps the most significant. In this study 6-amino-hexanoic acid was utilized as a model compound for lysinyl residues, the residues in the protein which are involved in the crosslinking reaction. A structure

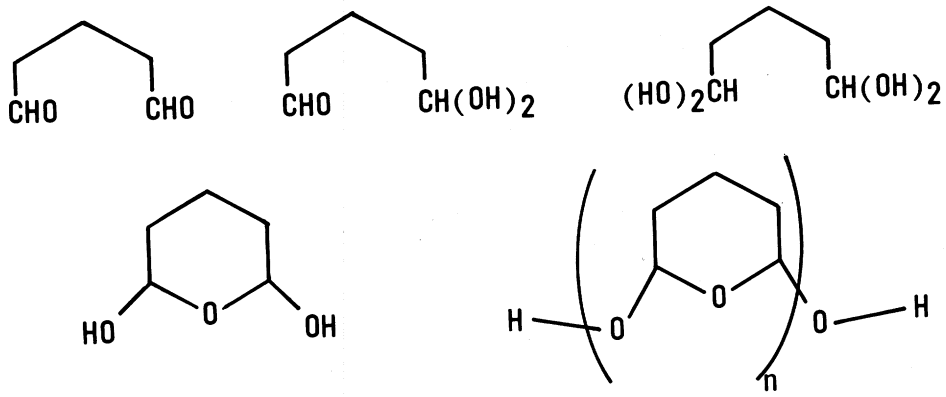


Figure 7. Glutaraldehyde Derivatives Present in Aqueous Solution.

proposed for a product Hardy et al. isolated and characterized had all of the spectroscopic and chromatographic characteristics of protein crosslinks. By analogy with the proposed structure for this product, it seems probable that the inital product is formed in the protein from a lysinyl residue and three molecules of glutaraldehyde which condense to form the heterocyclic ring compound as shown in Figure 8. The first step is the amine to aldehyde addition reaction which must be followed by aldehyde condensation reactions. At some point in the sequence, an oxidation step must be included to arrive at the pyridinium ring. Further reactions of the same types can then yield crosslinked products of various types, two of which are shown at the bottom of this figure.

Our studies (Korn, Feairheller, and Filachione, 1972) have shown that three products are isolatable from the reaction of glutaraldehyde with collagen, casein, polylysine, or α-N-carboxybenzoyl-lysine. The properties of two of these products are such that they could correspond to products like those shown here. However, it is not entirely clear why glutaraldehyde should be unique among the dialdehydes in forming stable crosslinks of this type in proteins. Succinaldehyde forms crosslinks, but they are not stable to hydrolysis, while adipaldehyde forms crosslinks only to a very limited extent (Cater, 1965). If these are the structures of the crosslinks, it is not obvious why one less or one more methylene group between the aldehyde groups is important to stability

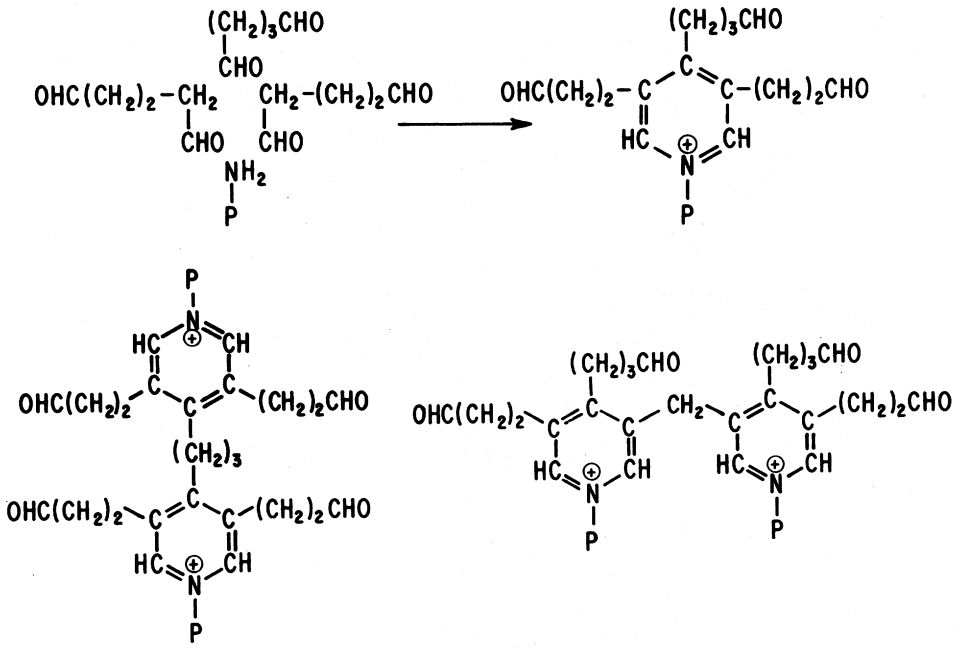


Figure 8. Reaction Products of Glutaraldehyde with Proteins.

or even crosslink formation. There appears to be continued interest in this problem and these studies will hopefully continue.

SUMMARY

From the limited information available, it appears that chemical crosslinking does occur as a result of tanning collagen with commercial chromium and aldehyde tanning agents. This, however, does not appear to be the case with vegetable tanning agents. The leather resulting from vegetable tanning has adequate stability for many uses without requiring chemical crosslinking. The other properties contributed by vegetable tanning materials are more important. Chromium tanning agents form relatively stable coordinate covalent bonds with collagen carboxyl groups, resulting in a product that has a good hydrothermal stability but is somewhat unstable to the action of acids and bases. Formaldehyde forms covalent bonds with collagen amino groups but they are unstable to hydrolytic conditions and the tannage is not stable. Glutaraldehyde, on the other hand, forms covalent crosslinks with collagen amino groups, and these crosslinks have a good hydrothermal

stability as well as being stable to the action of dilute acids and bases. For this reason, glutaraldehyde tannages are often used in conjunction with chrome tannages to impart perspiration resistance and washability to leathers. In spite of all of the extensive literature on tanning and tanning agents, serious gaps remain concerning the nature and extent of crosslinking and stabilization caused by all of these tanning agents. Additional research is needed to develop a better understanding of the reactions involved and the structures of the crosslinks formed. This understanding would lead to better utilization of existing tanning agents and development of new tanning agents.

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